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14. ABSTRACT: We proposed to test the possibility of using midkine promoter driven adenovirus (AdMk) to selectively kill neurofibroma cells. It is suggested from the literature that midkine is expressed at higher levels in cells and sera of NF1 patients. We initially obtained a version of the AdMk and tested its cytopathic effect on a neurofibroma cell line ST88. However a more recent publication indicated that this virus would be too toxic for normal cells as well. We then obtained from our new collaborator several different modified adenovirus and tested their cytopathic effects. Promoter activities of the midkine and Cox2 are also assayed in several cell lines.

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INTRODUCTION:

Neurofibromatosis type I (NF1) is a genetic disorder of the nervous system. Its clinical symptoms range from benign neurofibromas to malignant peripheral nerve sheath tumors (MPNST). Abnormalities in the levels of growth factors are often associated with tumorigenesis, which is also the case with NF1 tumors. Midkine is one of the growth factors that have been implicated in the stimulation of NF1 tumor growth [1]. During embryogenesis, this heparin binding growth factor is involved in neuronal development and expressed at the epithelial-mesenchymal interfaces [2]. In vitro, midkine is found to posses angiogenic, mitogenic, anti-apoptotic, and transforming activities, that are all pro-tumorigenic [2]. Its expression level is elevated in tumor or serum samples not only in NF1 patients but also in various other cancer patients [1-3]. Therefore, midkine could be a molecular target for cancer therapy including NF1. The regulatory region of the midkine gene has been examined and was found to activate reporter genes in immortalized cells [4]. Recombinant adenovirus has been constructed in which the adenovirus early gene E1A is driven by the midkine promoter (AdMk) [4]. Tumor cells were susceptible to AdMk whereas normal cells are resistant. In addition, AdMk treated HuH-7 cells no longer develops flank tumors when injected into immunodeficient SCID mice.

The level of the growth factor midkine is elevated in NF1 tumor patients, which is most likely to be at the transcription level. AdMk should be able to preferentially replicate in NF1 tumor cells. Conditionally replicative adenovirus driven by midkine promoter should be tested for its potential for NF1 therapy.

BODY:

Task 1. Verify the transcriptional activity of midkine promoter in various NF1 cell lines.

a. Perform luciferase assays using midkine promoter driven luciferase reporter in various NF1 cell lines

The mRNA level of midkine is higher in NF1 tumors and in the MPNST derived ST88-14 cells [1]. Presumably, the amount of transcription from the midkine promoter is higher in NF1 tumors and derived cells. To verify this assumption, we measured the transcriptional activity of the midkine promoter in various NF1 cell lines. We obtained three NF1 cell lines from Dr. Julian Downward [5]. A midkine promoter driven luciferase construct (Mk-luc) was obtained from Dr. Wildner. Mk-luc or a Gal4 promoter driven luciferase construct (G4-luc, as negative control) were cotransfected with a control renilar luciferase construct (for normalization of transfection efficiency) into NF1 cell lines. Dual luciferase assays were performed. We also chose two glioma cell lines, U87 and U251 as controls for the luciferase assays. These cell lines were used to test the cytotoxic effect of AdMk for glioma cell lines [6]. U87 has lower midkine promoter activity whereas U251 has higher activity. As shown in figure1, U251 has 10 fold higher midkine promoter activity compared to U87. ST88-14 and NF90-8 cells have 2-3 fold higher activity. (Figure1)

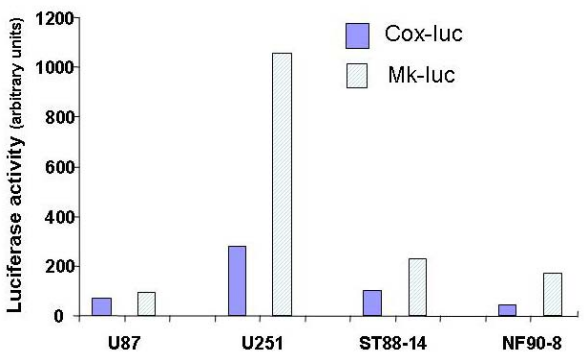


Figure1. Cox-2 and midkine promoter activity measured by luciferase assays. 10^5 cells were plated in 6-well dishes and 0.9 g Cox-luc or Mk-luc and 0.1 g renilar luciferase plasmid were transfected using Fugene6 reagent. Cells were harvested 48 hours post transfection and dual luciferase assays performed.

NF1 cells also have elevated Ras activity due to the genetic defect in the NF1 gene which encodes a negative regulator of Ras. Ras is known to up-regulate cyclooxygenase-2 (Cox-2) [7]. For reasons explained in the following report for Task2, we also obtained a Cox-2 promoter driven luciferase reporter (Cox-luc) and performed dual luciferase assays in the same cell lines. We found that the Cox-2 promoter activity is also higher in U251 cells compared to U87 but only marginally higher in NF1 cells (Figure1). Both U87 and U251 glioma cells have mutation thus loss of function for the PTEN, therefore PI3kinase, which is one of the major downstream effector of Ras, is hyperactive. It is possible that U87 already has a higher Cox-2 promoter activity compared to normal cells.

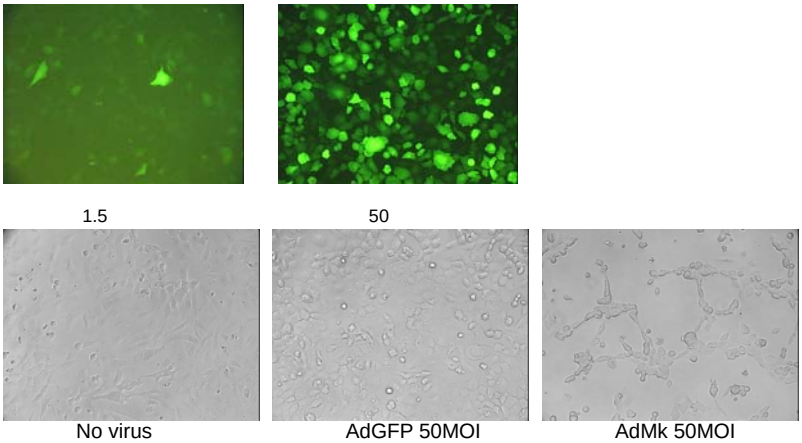
Task 2. Examine the cytotoxic effect of AdMk in NF1 cell lines.

a. Amplify and purify small scale AdMk and titer the virus.

AdMk was obtained from Dr. Tagawa from Japan [4]. We amplified the virus in 293E4 cells and prepared a medium scale purification using CsCl2. The virus prep was determined using a prep of wild type adenovirus of known concentration with an ELISA method. In late spring, we found a new publication reporting the generation of a set of adenoviruses where the E1 and the E4 promoters (singly or doubly) are replaced by midkine promter or cox2 promoter. The authors of this paper also tested the toxicity of these viruses. The AdMk like virus produced much more toxicity in keratinocytes compared to the double promoter replacement viruses. We requested and obtained those double viruses (AdCoxCox, AdCoxMk, AdMkCox, and AdMkMk), as well as luciferase reporters for these promoters. We amplified the virus again in 293E4 cells and prepared medium scale CsCl2 purifications and determined the titer.

b. Test the cytotoxic effect of AdMk versus control viruses in NF1 cell lines

Initially the cytotoxic effect of AdMk was tested in ST88-14 cells using AdGFP as a control. As shown in Figure2, ST88-14 cells are readily infectable by adenovirus indicated by the fluorescence of GFP following AdGFP infection but AdGFP has no cytotoxic effect on these cells. AdMk had a very strong cytotoxic effect on ST88-14 cells.



Although the results were encouraging, there were concerns regarding the toxicity of this particular AdMk virus in normal cells. First, this particular virus that we obtained from Dr. Tagawa has only an insertion of the midkine promoter in the E1 promoter which may result in a virus that still retains the majority of the wild type E1 promoter activity. Second, normal fibroblast was used to test the toxicity of this virus in the original publication. Adenovirus typically infects cells that are epithelial origin much more readily than fibroblasts.

As reported in Task2a, we found a more recent publication from Dr. Wildner's group in Germany describing engineered viruses that have both the E1 and E4 virus promoter replaced by various heterologous promoters including midkine promoter [8]. They showed that a virus similarly constructed as the AdMk we obtained from Dr. Tagawa has at least 67% toxicity in normal keratinocytes, whereas the double promoter replaced viruses had as low as only 4% lysis. We infected NF1 cell lines, the two glioma cell lines and also HMLE (an immortalized normal human mammary epithelial cell line) as control, with these new viruses and AdGFP (Figure3). As shown in Figure2, ST88-14 as well as U251 cells were killed by AdCoxCox, AdCoxMk, AdMkCox even at a low multiplicity of infection (MOI) of 0.8. NF90-8 and U87 cells are more resistant and were killed at a higher MOI of 12. AdGFP was not able to kill these cells. In contrast, there was very little cytotoxic effect of these adenoviruses on HMLE cells. 293E4 cells support the replication of all adenoviruses and serves as a control for the titer of the viruses. All of the cell lines are infectable by adenovirus as GFP expression can be observed after infection of AdGFP (not shown).

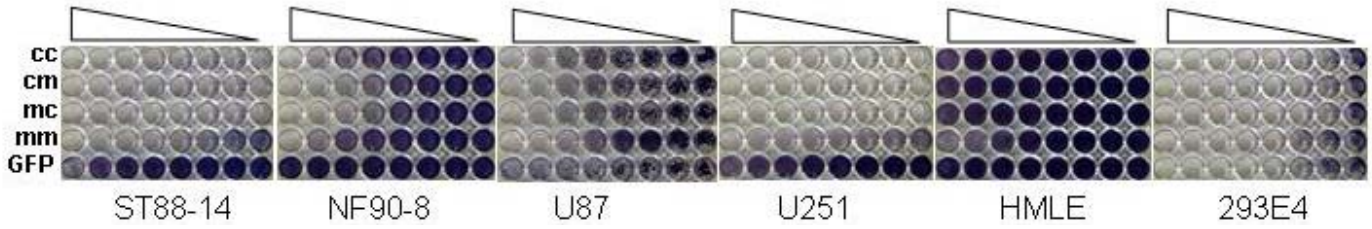


Figure2. Cytotoxic effect of adenoviruses on different cell line. $4x10^4$ cells were plated in 48 wells, next day infected with indicated viruses at serial dilutions in MEGM media (cc=AdCoxCox, cm=AdCoxMk, mc=AdMkCox, mm=AdMkMk, GFP=AdGFP). Serial dilutions of the viruses are from MOI of 100 to 0.006 at 4 fold dilutions each step for 193E4, and from MOI of 100 to 0.8 at 2 fold dilutions each step for all other cells. Cells were stained with 0.5% crystal violet solution after 4 days.

The following tasks have not been initiated due to the time spent on obtaining, amplifying and testing of the new set of viruses.

Task 3. Test the effect of AdMk on NF1 tumors in SCID mice.

a. Generate SCID mice with xenografted NF1 tumor
b. Test the effect of AdMk in mice models

KEY RESEARCH ACCOMPLISHMENTS:

- Determined midkine and Cox-2 promoter activities using dual luciferase assays in N1 cell lines.
- Obtained, amplified and purified 5 different recombinant adenovirus.
- Tested the cytotoxic effects of these viruses in NF1 cell lines, glioma cell lines and normal human epithelial cell lines.

REPORTABLE OUTCOMES:

Amanuel Osmamaw Kenebe, a graduate student in San Francisco State University participated in this project and is now applying for PhD programs.

CONCLUSIONS:

We successfully obtained various recombinant adenovirus where the E1 and E4 promoters were replaced by tumor specific promoters. The levels of midkine and Cox-2 promoter activitites were measured in NF1 cells and found to be modestly elevated. These recombinant viruses seem to be able to selectively kill NF1 cells in a replication competent manner.

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